

### III. CONCLUSION

21. From the foregoing analysis, I find that Spezyme Ethyl contains DNA having the nucleotide sequence set forth in Exhibit 3. This DNA encodes a protein, which has the amino acid sequence that is also depicted in Exh. 3.

22. I declare under penalty of perjury pursuant to the laws of the United States of America that the foregoing statements are true and correct.

Dated: 19 May 2005

Respectfully submitted,

Steen T. Jørgensen  
Steen Troels Jørgensen

#### Attachments:

Exhibit 1: *Curriculum Vitae* of Steen Troels Jørgensen

Exhibit 2: Spezyme Ethyl DNA Sequence

Exhibit 3: P.L. Jørgensen *et al.*, "Cloning of a chromosomal  $\alpha$ -amylase gene from *Bacillus stearothermophilus*" *FEMS Microbiology Letters* (1991) 77:271-276.

# **EXHIBIT 1**

## Steen Troels Jørgensen

### Curriculum Vitae

<b>Initials:</b> STJq	<b>Full name:</b> Steen Troels Jørgensen
<b>Date of birth:</b> 8. June 1958	<b>Reports to (initials):</b> MDRa
<b>Year of graduation:</b> 1985	<b>University/college:</b> University of Copenhagen
<b>Degree:</b> Cand. Scient	<b>Joined NN in year:</b> 1985

#### Job Experience:

Year:	Business unit/company:	Position:	Assignments/tasks:
1985	Bacterial Gene Technology, Novo Industri A/S.	Research Scientist	Gene technology as used in the construction of bacterial production strains for high-level expression of enzymes, and in the cloning and expression of novel enzyme coding genes.
1995	Bacterial Gene Technology, Enzyme Business, Novo Nordisk A/S	Principal Scientist (Science Manager)	As above.
2002	Bacterial Gene Technology, Novozymes A/S	Senior Science Manager	As above.

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## **EXHIBIT 2**



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## Cloning of a chromosomal $\alpha$ -amylase gene from *Bacillus stearothermophilus*

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Received 22 August 1990  
Accepted 18 September 1990

Key words: Native plasmids; Heat stable amylases; Nucleotide sequence; Evolution; Cloning in *Bacillus subtilis*

### 1. SUMMARY

We have cloned and sequenced a gene for a heat-stable  $\alpha$ -amylase from a natural isolate of *Bacillus stearothermophilus*. Previously, it had been shown that *B. stearothermophilus* amylase genes may be harboured on indigenous plasmids. We have found that our isolate harbours the amylase gene only on the chromosome and not on its indigenous plasmid.

### 2. INTRODUCTION

Amylases are exoenzymes, which are widely distributed in nature. All amylases, which have been sequenced so far, share significant homology suggesting an evolutionary relationship. The observation that an  $\alpha$ -amylase gene from *B. stearothermophilus* is harboured on a natural plasmid [1] supports the notion that gene transfer can take place between related strains. In this paper we show that homologous amylase genes

can reside either on a native plasmid or on the chromosome of different strains of *B. stearothermophilus*.

### 3. MATERIALS AND METHODS

#### 3.1. Bacterial strains and plasmids

*Escherichia coli* K-12 MT102 is (*araD139*,  $\Delta$  (*ara-leu*)7697, *lacX74*, *galU*, *galK*, *rpsL*,  $r^- m^+$ ). *Bacillus subtilis* DN1885 (*amyE*, *amyR2*) is a derivative of *B. subtilis* 168 strain RUB200 [2]. *B. stearothermophilus* DN1792 and DN1793 were isolated from a household hot water system and classified by Deutsche Sammlung für Mikroorganismen. ATCC 31783 is a *B. stearothermophilus* from the American Type Culture Collection.

Plasmid pUN121 has been described [3]. Plasmid pDN2360 was constructed by ligation of *Pst*I fragments from pUN121 and plasmid pDN1380 [4] (Fig. 1). Plasmid pBB37 (Fig. 1) was constructed by inserting a *Cla*I fragment harbouring the gene for an  $\alpha$ -amylase from *B. amyloliquefaciens* [5] into the *Cla*I site of plasmid pDN1620, which was derived from pDN1380 by insertion of an oligonucleotide linker in the *Eco*RI site.

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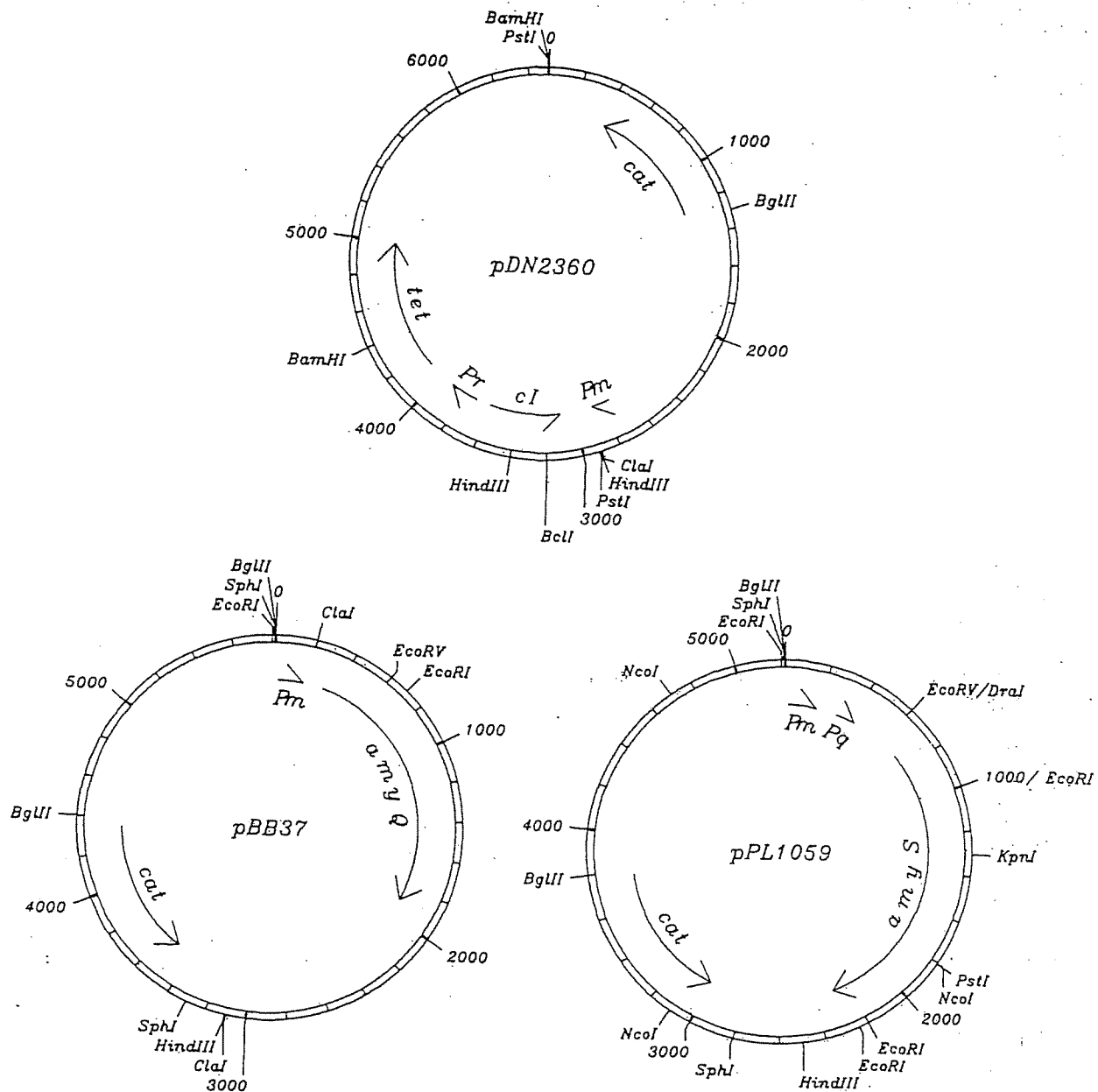


Fig. 1. Restriction maps of plasmids. pDN2360 replicates in both *E. coli* and in *B. subtilis*. pBB37 and pPL1059 replicate in *B. subtilis*. *cI* is the coliphage  $\lambda$  repressor gene.  $P_r$  is a promoter from  $\lambda$ .  $P_m$  is the promoter of the maltogenic amylase gene *amyM* [4]. *cat* is the chloramphenicol resistance conferring gene from plasmid pC194 of *Staphylococcus aureus*. *amyQ* is the amylase gene from *B. amyloliquefaciens* [5].  $P_q$  is the promoter from the *amyQ* gene. *amyS* is the amylase gene from *B. stearotheophilus* described in this work.

### 3.2. Transformation and plasmid preparation

Competent cells, transformation, and preparation of recombinant plasmids were as described

[4]. Transformants of *E. coli* were selected with 7  $\mu\text{g/ml}$  tetracycline. Transformants of *B. subtilis* were selected with 6  $\mu\text{g/ml}$  chloramphenicol.

10 20 30 40 50 60 70 80 90 100 110 120 130 140 150 160 170 180 190 200 210 220 230 240 250 260 270 280 290 300 310 320 330 340 350 360 370 380 390 400 410 420 430 440 450 460 470 480 490 500 510 520 530 540 550 560 570 580 590 600 610 620 630 640 650 660 670 680 690 700 710 720 730 740 750 760 770 780 790 800 810 820 830 840 850 860 870 880 890 900 910 920 930 940 950 960 970 980 990 1000 1010 1020 1030 1040 1050 1060 1070 1080 1090 1100 1110 1120 1130 1140 1150 1160 1170 1180 1190 1200 1210 1220 1230 1240 1250 1260 1270 1280 1290 1300 1310 1320 1330 1340 1350 1360 1370 1380 1390 1400 1410 1420 1430 1440 1450 1460 1470 1480 1490 1500 1510 1520 1530 1540 1550 1560 1570 1580 1590 1600 1610 1620 1630 1640 1650 1660 1670 1680 1690 1700 1710 1720 1730 1740 1750 1760 1770 1780 1790 1800 1810 1820 1830 1840 1850 1860 1870 1880 1890 1900 1910 1920 1930 1940 1950 1960 1970 1980 1990 2000 2010 2020 2030 2040 2050 2060 2070 2080 2090 2100 2110 2120 2130 2140 2150 2160 2170 2180 2190 2200 2210 2220 2230 2240 2250 2260 2270 2280 2290 2300 2310 2320 2330 2340 2350 2360 2370 2380 2390 2400 2410 2420 2430 2440 2450 2460 2470 2480 2490 2500 2510 2520 2530 2540 2550 2560 2570 2580 2590 2600 2610 2620 2630 2640 2650 2660 2670 2680 2690 2700 2710 2720 2730 2740 2750 2760 2770 2780 2790 2800 2810 2820 2830 2840 2850 2860 2870 2880 2890 2900 2910 2920 2930 2940 2950 2960 2970 2980 2990 3000 3010 3020 3030 3040 3050 3060 3070 3080 3090 3100 3110 3120 3130 3140 3150 3160 3170 3180 3190 3200 3210 3220 3230 3240 3250 3260 3270 3280 3290 3300 3310 3320 3330 3340 3350 3360 3370 3380 3390 3400 3410 3420 3430 3440 3450 3460 3470 3480 3490 3500 3510 3520 3530 3540 3550 3560 3570 3580 3590 3600 3610 3620 3630 3640 3650 3660 3670 3680 3690 3700 3710 3720 3730 3740 3750 3760 3770 3780 3790 3800 3810 3820 3830 3840 3850 3860 3870 3880 3890 3900 3910 3920 3930 3940 3950 3960 3970 3980 3990 4000 4010 4020 4030 4040 4050 4060 4070 4080 4090 4100 4110 4120 4130 4140 4150 4160 4170 4180 4190 4200 4210 4220 4230 4240 4250 4260 4270 4280 4290 4300 4310 4320 4330 4340 4350 4360 4370 4380 4390 4400 4410 4420 4430 4440 4450 4460 4470 4480 4490 4500 4510 4520 4530 4540 4550 4560 4570 4580 4590 4600 4610 4620 4630 4640 4650 4660 4670 4680 4690 4700 4710 4720 4730 4740 4750 4760 4770 4780 4790 4800 4810 4820 4830 4840 4850 4860 4870 4880 4890 4900 4910 4920 4930 4940 4950 4960 4970 4980 4990 5000 5010 5020 5030 5040 5050 5060 5070 5080 5090 5100 5110 5120 5130 5140 5150 5160 5170 5180 5190 5200 5210 5220 5230 5240 5250 5260 5270 5280 5290 5300 5310 5320 5330 5340 5350 5360 5370 5380 5390 5400 5410 5420 5430 5440 5450 5460 5470 5480 5490 5500 5510 5520 5530 5540 5550 5560 5570 5580 5590 5600 5610 5620 5630 5640 5650 5660 5670 5680 5690 5700 5710 5720 5730 5740 5750 5760 5770 5780 5790 5800 5810 5820 5830 5840 5850 5860 5870 5880 5890 5900 5910 5920 5930 5940 5950 5960 5970 5980 5990 6000 6010 6020 6030 6040 6050 6060 6070 6080 6090 6100 6110 6120 6130 6140 6150 6160 6170 6180 6190 6200 6210 6220 6230 6240 6250 6260 6270 6280 6290 6300 6310 6320 6330 6340 6350 6360 6370 6380 6390 6400 6410 6420 6430 6440 6450 6460 6470 6480 6490 6500 6510 6520 6530 6540 6550 6560 6570 6580 6590 6600 6610 6620 6630 6640 6650 6660 6670 6680 6690 6700 6710 6720 6730 6740 6750 6760 6770 6780 6790 6800 6810 6820 6830 6840 6850 6860 6870 6880 6890 6900 6910 6920 6930 6940 6950 6960 6970 6980 6990 7000 7010 7020 7030 7040 7050 7060 7070 7080 7090 7100 7110 7120 7130 7140 7150 7160 7170 7180 7190 7200 7210 7220 7230 7240 7250 7260 7270 7280 7290 7300 7310 7320 7330 7340 7350 7360 7370 7380 7390 7400 7410 7420 7430 7440 7450 7460 7470 7480 7490 7500 7510 7520 7530 7540 7550 7560 7570 7580 7590 7600 7610 7620 7630 7640 7650 7660 7670 7680 7690 7700 7710 7720 7730 7740 7750 7760 7770 7780 7790 7800 7810 7820 7830 7840 7850 7860 7870 7880 7890 7900 7910 7920 7930 7940 7950 7960 7970 7980 7990 8000 8010 8020 8030 8040 8050 8060 8070 8080 8090 8100 8110 8120 8130 8140 8150 8160 8170 8180 8190 8200 8210 8220 8230 8240 8250 8260 8270 8280 8290 8300 8310 8320 8330 8340 8350 8360 8370 8380 8390 8400 8410 8420 8430 8440 8450 8460 8470 8480 8490 8500 8510 8520 8530 8540 8550 8560 8570 8580 8590 8600 8610 8620 8630 8640 8650 8660 8670 8680 8690 8700 8710 8720 8730 8740 8750 8760 8770 8780 8790 8800 8810 8820 8830 8840 8850 8860 8870 8880 8890 8900 8910 8920 8930 8940 8950 8960 8970 8980 8990 9000 9010 9020 9030 9040 9050 9060 9070 9080 9090 9100 9110 9120 9130 9140 9150 9160 9170 9180 9190 9200 9210 9220 9230 9240 9250 9260 9270 9280 9290 9300 9310 9320 9330 9340 9350 9360 9370 9380 9390 9400 9410 9420 9430 9440 9450 9460 9470 9480 9490 9500 9510 9520 9530 9540 9550 9560 9570 9580 9590 9600 9610 9620 9630 9640 9650 9660 9670 9680 9690 9700 9710 9720 9730 9740 9750 9760 9770 9780 9790 9800 9810 9820 9830 9840 9850 9860 9870 9880 9890 9900 9910 9920 9930 9940 9950 9960 9970 9980 9990 10000

Fig. 2. DNA sequence of the *anyS* gene. Promoter regions -35 and -10 and a ribosome binding site have been underlined starting at bp 11, 35 and 140, respectively. An arrow shows the processing site after the 34 amino acid signal peptide.

Large plasmids were prepared by alkaline lysis according to ref. 6 with the exception that 50 mM glucose was added to the suspension buffer and that the samples were centrifuged for only 10 min before phenol extraction. Chromosomal DNA was prepared by phenol extraction [7].

### 3.3. DNA sequencing

DNA sequencing was performed directly on doublestranded plasmid templates using the Sequenase™ kit from United States Biochemical Corporation.

### 3.4. Southern analyses

Chromosomal and plasmid DNA was digested with restriction enzymes, fractionated on 0.7% agarose gels and blotted onto nitrocellulose filters. pPL1022 was labelled by nick translation using [<sup>32</sup>P]dCTP (Amersham). The hybridization was according to Southern [8].

## 4. RESULTS

### 4.1. Cloning in *E. coli*

Genomic DNA from strain DN1792 was partially digested with *Sau*3A and fragments, of 3–10 kb were ligated with the *Bcl*I digested selection vector pUN121. *E. coli* MT102 was transformed with the ligation mixture selecting for tetracycline resistance on agar plates containing starch. Of 10000 transformants, two had a clear amylase positive phenotype. One of these contained a plasmid, pPL1011, with an insert of 8 kb. pPL1011 was partially digested with *Sau*3A and ligated with selection vector pDN2360 digested with *Bcl*I. Tetracycline resistant transformants of *E. coli* MT102 were screened for amylase expression and one positive transformant harbouring pPL1022 with an insert of 2.2 kb was kept for further study. The structural gene for the *B. stearothermophilus* amylase was called *amyS*.

### 4.2. Cloning in *B. subtilis*

A 2.0 kb *Dra*I-*Hind*III fragment of pPL1022 was ligated with the 3.3 kb fragment of *B. subtilis* plasmid pBB37 and transformed into *B. subtilis* DN1885 selecting for chloramphenicol resistance

and screening for amylase activity. A positive transformant harboured the 5.3 kb plasmid pPL1059 (Fig. 1).

### 4.3. DNA sequence analysis

The sequence of the *amyS* gene of pPL1022 is shown in Fig. 2. Of the 1814 bp of this sequence, 33 bp (1.8%) differed from the sequence of Nakajima et al. [9] and 40 bp (2.2%) from the sequence of Gray et al. [10]. Comparing the amino acid sequence of the mature enzyme and the signal peptide with the above sequences showed 12 (2.2%) and 9 (1.6%) differences, respectively. Between the sequences of Nakajima et al. [9] and Gray et al. [10], there are 13 (2.4%) amino acid differences.

At the amino acid level, one of the three sequences differs from the two others at 17 positions of which 4 (11.8%) are in the signal peptide of 34 amino acids and the remaining 13 (2.5%) in the mature part of 515 amino acids.

### 4.4. Southern blotting analysis

To test the localization in *B. stearothermophilus* of the cloned amylase gene, plasmids were prepared from the *B. stearothermophilus* DN1792, DN1793 and ATCC 31783 [11]. DN1792 and DN1793 contained each one plasmid of 25 kb and ATCC 31783 contained three plasmids of 12, 15 and 26 kb as reported by Mielenz [1] (Fig. 3A). Using the cloned amylase gene as probe, hybridization was only seen with the 26 kb plasmid of ATCC 31783. The probe, however, clearly hybridized with the chromosomal DNA of DN1792 and DN1793 (Fig. 3B). Thus, *amyS* is located on the chromosome of DN1792 and DN1793 and on a native plasmid in ATCC 31783.

## 5. DISCUSSION

The *amyS* gene of a natural isolate of *B. stearothermophilus* has been cloned, sequenced and compared with genes from other *B. stearothermophilus* strains. The sequences of three independently isolated *amyS* genes (refs. 9, 10, and this work) show some variations in particular in the signal peptide regions.

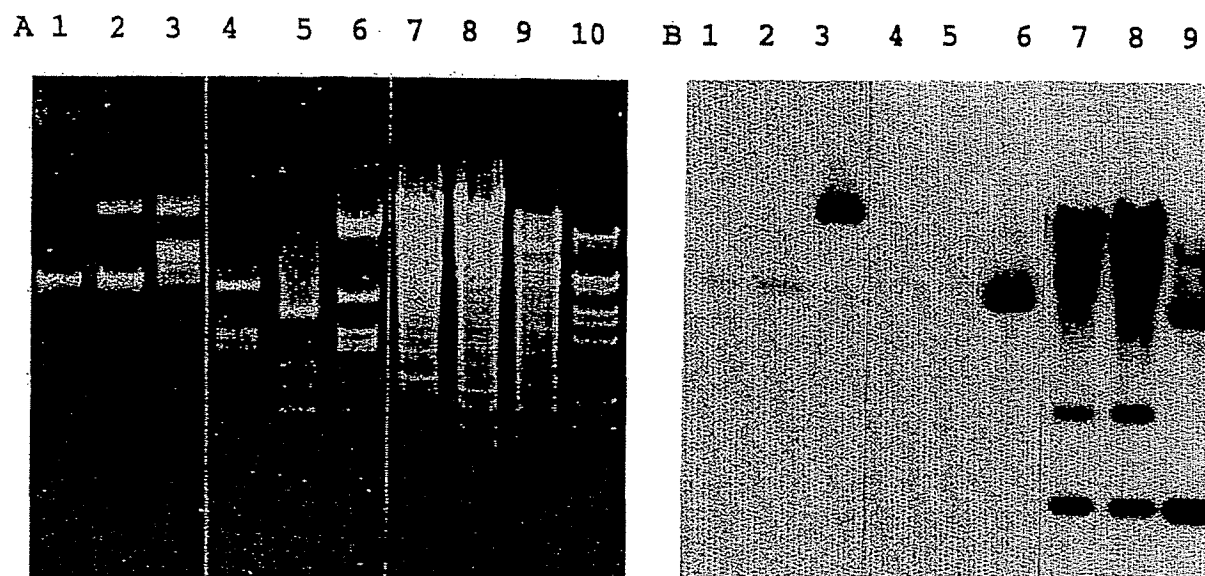


Fig. 3. Southern blotting analysis of *B. stearothermophilus* plasmids and chromosomal DNA. (A) 0.7% agarose gels stained by ethidium bromide. (B) The same gels as in A but hybridized with nicktranslated pPL1022. Lanes 1 and 4, plasmid from DN1792; lanes 2 and 5, plasmid from DN1793; lanes 3 and 6, plasmid from ATCC 31783; lanes 7-9, chromosomal DNA from DN1792, DN1793 and ATCC31783, respectively; lane 10,  $\lambda$  DNA digested by *BstE2*. Lanes 1-3, uncut plasmids; Lanes 4-6, plasmids cut by *HindIII*; Lanes 7-9, chromosomal DNA cut by *EcoRI*.

Satoh et al. [12] have cloned two closely related amylase genes from what appear to be two phylogenetically very distant *B. stearothermophilus* strains. Nucleotide sequence analyses show that the regions flanking the amylase genes are completely different. Sen and Ariel [13] report on the location of amylase genes in two *B. stearothermophilus* strains. In one strain, the amylase gene is present both on two different indigenous plasmids and in multiple copies on the chromosome. In the other strain, multiple copies were found on the chromosome but no indigenous plasmid could be detected. We speculate that the multiple chromosomal genes in these two strains may be the result of plasmid integration. In this paper, it is shown that two other *B. stearothermophilus* strains harbour the amylase gene on the chromosome only, despite the presence of indigenous plasmids. It is thus likely that there is a mechanism in *B. stearothermophilus* which allows for migration of amylase genes from chromosome to plasmid or vice versa.

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